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Sensitivity of *Botrytis cinerea* Isolates Collected from Strawberry to SDHI Fungicide Boscalid

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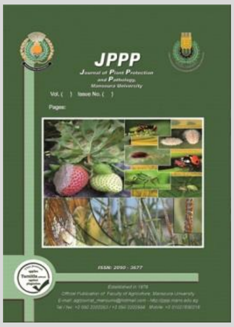

ABSTRACT

Egypt is considered one of the main strawberry producers countries. Grey mould, caused by *Botrytis cinerea*, is a destructive disease resulting in significant losses in strawberry production worldwide as fruits might be infected in the field, storage and transport. Management approaches for the pathogen in strawberries depend mainly on using protective fungicides. Recently, lack of boscalid fungicide efficiency reported in many countries. The current study aims to detect and monitor *B. cinerea* resistance to boscalid in the main strawberry production governorates in Egypt (Beheira, Ismailia and Qalyubie) between 2019 and 2021. 269 isolates were collected and tested to distinguish resistant isolates. Sensitivity of a set of resistant and sensitive isolates was measured by determining the effective concentration that inhibits 50 % of mycelial growth. The results showed that, 225 (83.6%) isolates were resistant to boscalid while, only 44 (16.4%) isolates were sensitive. Monitoring of resistance frequency revealed that there were remarkable increases in resistance frequencies to boscalid in *B. cinerea* isolates during 2019 and 2021 seasons as it was 84.03% in 2019 increased to 98.87% in 2021. 17 sensitive isolates randomly selected were used to determine the EC_{50} for boscalid using mycelial growth inhibition assay and the EC_{50} values ranged from 0.0067 to 1.14 µg/ml with a mean of 0.29 µg/ml. While, the EC_{50} values for17 resistant isolates randomly selected ranged from 2.1 to 16.2 µg/ml with a mean of 7.3 µg/ml. Among 225 boscalid resistant isolates, 192 (85.33%) isolates showed resistant to pyraclostrobin which may lead to failure of gray mold control in case of usage of those fungicides mixture in control strategies.

Keywords: *Botrytis cinerea*, Strawberry, SDHI, Boscalid, Resistance

INTRODUCTION

Strawberry is an important horticultural crop, widely grown in all temperate regions in the world. Gray mold, caused by *Botrytis cinerea* (*B. cinerea*), is among the most important diseases of several crops as it can infect over than 200 plant species including strawberry, causing significant yield losses worldwide. *B.cinerea* found in the remains of the botanical parts. High humidity helps develop disease. With the start of flowering, fungi attack flowers, which lead to less flowers and loss of fruits (Adnan *et al* 2019).

Control of gray mold in strawberry fields mainly depends on usage of protective fungicides extensively in order to avoid the infection starting process. Recently, growers are facing a real challenge due to less efficiency of common fungicides used as a result of rapid development of *B. cinerea* resistant populations (Amiri *et al* 2013). Resistance to multiple chemical classes of fungicides, such as Quinone outside inhibitors (QoIs), Hydroxyanilides (HAs) or Phenylpyrroles (PPs) reported in many studies (Bardas *et al* 2010 and Fan *et al* 2017) complicated the control process and lead to failure in the strategies used to minimizing the losses. Succinate dehydrogenase inhibitors (SDHIs) also known as succinate: quinone oxidoreductase or succinate dehydrogenase such as boscalid is wide spectra fungicides used recently in *B. cinerea* control. According to FRAC SDHI fungicides are classified as medium to high risk of resistance development. Boscalid was the first molecule belonging to the SDHIs that was introduced for the control of *B. cinerea*. The target of the SDHI fungicides is inhibiting complex II in the mitochondrial respiratory chain, leading finally to blocking the cell energy production. *B. cinerea* boscalid resistant populations were detected in many countries worldwide. Wang *et al* (2021) mentioned that 45.16% of *B. cinerea* isolates were resistant to boscalid. Similarly, Cosseboom *et al* (2019) stated that 24% of isolates were resistant to the fungicide. Additionally, monitoring the resistance frequency is very important in order to prolong the effective use of the fungicide. Cui *et al* (2021) tested the sensitivity of *B. cinerea* isolates to boscalid and the results illustrated that the resistance frequency increased from 5.26% and 0 to 56.25% and 52% for Laiwu and Liaocheng locations respectively, after five years of intensive usage of this fungicide. Resistance to boscalid linked to several mutations in one of the subunits (B, C, or D) of the SDH complex (Hu *et al* 2016).

Using of fungicides combinations is an effective strategy to delay the development of resistant populations. Boscalid and quinone outside inhibitor (QoI) fungicide pyraclostrobin mixture used recently to control *B. cinerea* in Egypt widely. Worldwide Pristine® is a combination of the SDHI and QoI fungicides used in the control of gray mold (kim and Xiao 2010 and Fernández-Ortuño *et al* 2012).

Therefore, the objectives of the current study were to detect boscalid resistance frequencies in *B. cinerea* isolates collected from different governorates in Egypt, monitor resistance to SDHI fungicide in *B. cinerea* from strawberry

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fields and to determine the effective concentrations EC_{50} of the sensitive and resistant isolates. Moreover, to investigate the dual resistance between boscalid and pyraclostrobin.

MATERIALS AND METHODS

Fungal isolates and culture conditions:-

In this study, 269 *B. cinerea* isolates were collected between 2019 and 2021. The governorates selected for isolates collection were Beheira, Ismailia and Qalyubie. Each governorate 4 commercial annual strawberry fields were chosen. Strawberry fruit carrying typical symptoms of gray mold obtained from commercial fields; one fruit picked from a different plant. Isolates of *B. cinerea* were cultured on potato dextrose agar medium (200 ml of potato juice originated from 200 g of potato, 20 g of agar and 20 g of dextrose, distilled water to 1 L) at 20°C in darkness. Each year, the isolation and purification of the fungal strains was done using a single hyphal tip method. The isolates slants were preserved in 15-mL plastic tubes containing PDA medium at 4°C until use.

Active ingredients: -

Technical grade boscalid (96% a.i.; Zhejiang Heben Pesticide & Chemicals Co., Ltd). Technical grade pyraclostrobin (98% a.i.; Shanghai Heben-Eastsun Medicaments Co,. Ltd.) The fungicides dissolved in 100% acetone to produce a stock solution containing 100 mg/ml.

According to previous studies, Hu *et al* (2016), Chen *et al* (2016) and Fan *et al* (2017) 75 µg/ml was used as a discriminatory concentration (a concentration that fully inhibits mycelial growth of the sensitive isolates) to distinguish boscalid resistant isolates from sensitive isolates. 5 mm in diameter mycelial plugs were separated with a cork borer from 3 day old colony margin of each isolate and placed upside down onto the media amended with fungicide. Colony diameter measured after 3 days at 22°C. For each fungicide, the isolates considered resistant if they can grow on the media amended with fungicide, while isolates cannot grow on the amended media were considered to be sensitive isolates.

Sensitivity of *B. cinerea* to boscalid:-

The sensitivity to boscalid was determined by evaluating the effective concentration resulting in inhibition of 50% of mycelial growth (EC_{50}) values for 17 boscalid sensitive (bos^S) and 17 boscalid resistant (bos^R) isolates. Technical grade boscalid (96% active ingredient [a.i.] was dissolved in100% acetone, adjusted to a concentration of 100 mg/ml, and added to PDA to produce final concentrations at (0, 0.1, 1, 2, 4 and 8 µg/ml) and (0, 10, 25, 50, 75 and 100 µg/ml) to test sensitive isolates and resistant isolates, respectively. The experiment was repeated twice. Data Processing System (DPS) program developed by Hangzhou Reifeng Information Technology Ltd., Hangzhou, China used to calculate the EC_{50} value for each isolate. The average of EC_{50} values from the two experiments for each isolate used in the analysis of the data as there was no significant difference ($P>0.05$) between the two experiments (Hamada *et al* 2011).

Dual resistance between pyraclostrobin and boscalid

To screen dual resistance in *B. cinerea* isolates between pyraclostrobin and boscalid, all boscalid resistant isolates were tested for sensitivity to pyraclostrobin. Discriminatory concentration of 10 µg/ml used to detect Pyraclostrobin resistant isolates also salicyl hydroxamic acid (99% a.i.; Sigma-Aldrich, St. Louis) at 100 µg/ml added to potato dextrose agar (PDA) to inhibit the alternative oxidase respiration.

RESULTS AND DISCUSSION

Resistance in *B. cinerea* isolates to SDHI fungicide boscalid

Among 269 isolates were collected, 225 (83.6%) isolates were resistant to boscalid fungicide as they were able to grow in media amended with discriminatory concentrations 75 ppm while, only 44 (16.4%) isolates were sensitive due to high selection pressure occurred. Moreover, it was observed that the percentage of resistance to boscalid was 84.03% in 2019 while it decreased in 2020 to 68% and again increased in 2021 to 98.87%.

In harmony with our results, high resistance frequencies reported by many authors such as Fernandez *et al.*, 2017 in Spain who mentioned that the percentage of resistance to boscalid in *B. cinerea* isolates collected from strawberries recorded was 73%. Also, Chen *et al* (2016) found that 66.67% of *B.cinerea* isolates collected were resistant to boscalid. While moderate resistance frequencies were reported by Weber *et al*., 2011 in Northern German who reported that 21.5% of isolates were resistant to boscalid in the isolates from strawberry. On the other hand, Yin *et al*., 2018 stated that the percentage of resistance to boscalid was only 4.3% among the isolates collected from nectarine and cherry in China. According to our results, there was remarkable increase in resistance frequencies to boscalid in *B. cinerea* isolates during 2019 and 2021 seasons as it was 84.03% in 2019 and increased to 98.87% in 2021. However, the resistance frequency decreased in 2020 to 68% which could be returned to the decrease in selection pressure occurred as a result of usage of different fungicides in this season or due to climatic changes. Many authors monitored the increasing and decreasing of the percentage of resistance in *B. cinerea* isolate to boscalid such as Rupp *et al*., 2016 in Germany who observed that the percentage of resistance to boscalid remarkably increased between 2001 to 2014 from 4% to 35%. In the same way, Leroch *et al*., 2011 found that frequencies of resistance to boscalid increased gradually among *B. cinerea* isolates collected from grap during the study as it was 2% in 2006 while it 26.7% in 2009. Also, Fernández-Ortuño *et al*., 2014 observed that percentage of resistance to boscalid was 29% in 2013 while it was 5% in 2012.

Table 1. Information about the sensitivity of 269 *Botrytis cinerea* isolates collected from strawberry in Egypt during three years from 2019 to 2021 to boscalid

Location	Year of collection	No. of isolates	No. of Bos^S	No. of Bos^R	% of resistance
Beheira	2019	37	3	34	91.9
Ismailia	2019	43	13	30	69.8
Qalyubie	2019	39	3	36	92.3
Beheira	2020	26	4	22	84.6
Ismailia	2020	26	12	14	53.8
Qalyubie	2020	23	8	15	65.2
Beheira	2021	25	Zero	25	100
Ismailia	2021	25	Zero	25	100
Qalyubie	2021	25	1	24	96
Total	/	269	44	225	
Resistant percentage%			16.4	83.6	

Bos^S boscalid sensitive Bos^R boscalid resistant

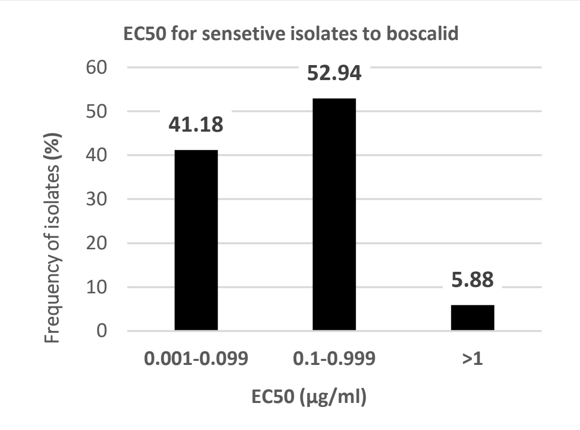
Sensitivity of *B. cinerea* isolates to boscalid:-

Among 269 isolates of *B. cinerea* obtained in the study, 17 Bos^S isolates randomly selected were used to determine the EC_{50} for boscalid using mycelial growth inhibition assays. The EC_{50} values of boscalid ranged from 0.0067 to 1.14 µg/ml with a mean of 0.29 µg/ml and the highest values EC_{50} of isolates (52.94%) ranged from 0.1 to 0.9 µg/ml (Fig.1). While, to determine the EC_{50} values for resistant isolates 17 Bos^R isolates randomly selected and tested using mycelial growth inhibition assays. The EC_{50} values ranged from 2.1 to 16.2 with a mean of 7.3 µg/ml. and the highest EC_{50}values of isolates (41.18%) ranged from 1 to 4.9 µg/ml (Fig.1)

RF (Resistance Factor) values to boscalid calculated by dividing the EC_{50} value of the resistant isolate by the mean EC_{50} value of the sensitive isolates (Table2) and the results illustrated that the resistant factor ranged between 7.26 to 55.8 which cleared that different levels of resistance could be found.

Table 2. EC_{50} of *Botrytis cinerea* (sensitive and resistant) isolates randomly selected to Boscalid:

Boscalid (EC_{50})				
Sensitive		Resistant		
Isolate	EC50	Isolate	EC50	RF
S 108	0.0068 ± 0.0002	8 B	2.1066 ± 0.0004	7.26
S 29	0.0305 ± 0.0003	S 3	2.5207 ± 0.0099	8.69
39	0.0316 ± 0.0002	BN 80	2.7407 ± 0.0003	9.45
13	0.0397 ± 0.0002	H4(1)	3.2938 ± 0.0002	11.36
BN 2	0.0728 ± 0.0001	36	3.9833 ± 0.0006	13.74
S 43	0.0737 ± 0.0002	MB 33	4.0720 ± 0.0009	14.04
S 35	0.0812 ± 0.0002	1	4.7259 ± 0.0013	16.29
19 B	0.1163 ± 0.0003	121	6.0356 ± 0.0006	20.81
H4(32)	0.1191 ± 0.0002	H3(6)2	6.1872 ± 0.0006	21.34
H4(19)	0.1313 ± 0.0002	MB 38	7.7608 ± 0.0010	26.76
S 137	0.1984 ± 0.0002	S 147	7.8016 ± 0.0006	26.9
S 28	0.2771 ± 0.0003	7	8.1572 ± 0.0005	28.13
48	0.3767 ± 0.0002	1 B	8.9026 ± 0.0009	30.69
21 B	0.6477 ± 0.0002	BN 105	10.8557 ± 0.2003	37.75
S 37	0.7085 ± 0.0002	H4(35)	14.0823 ± 0.1109	48.38
S 159	0.9676 ± 0.0002	H2(12)2	15.1577 ± 0.2007	51.99
49	1.1397 ± 0.0002	132	16.2061 ± 0.1599	55.8



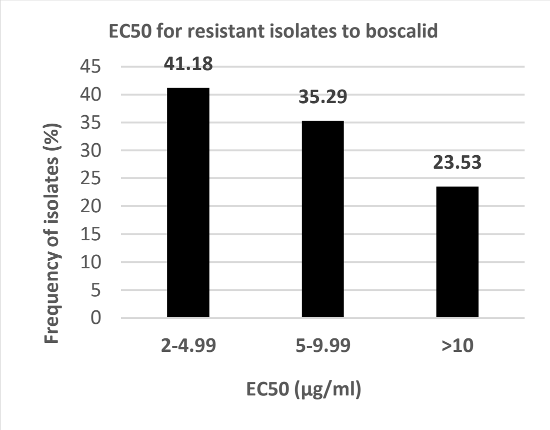


Fig 1. Sensitivity of *B. cinerea* isolates to boscalid

Our results were in harmony with many authors such as Chatzidimopoulos *et al* (2013) they studied the sensitivity of *B. cinerea* to boscalid by the determination of EC_{50} values and the results showed that the EC_{50} ranged from 0.38 to 2.7 mg /L using mycelial growth inhibition assay. Also, Pokorny *et al* (2016) in California reported that the effective concentration values of boscalid for *B. cinerea* sensitive isolates were 0.13 and 0.29 ppm while it was more than 100 ppm for resistant isolates.

Resistance to pyraclostrobin and boscalid

The results obtained in the current study indicated that among 225 boscalid resistant isolates detected 192 (85.33%) isolates showed resistance to pyraclostrobin which indicated that using of a mixture of both fungicides may result in the failure of the pathogen control. In harmony with our results, Fernández-Ortuño *et al* (2012) reported that 61.5% of *B. cinerea* isolates tested were resistant to both pyraclostrobin and boscalid. Moreover, kim and Xiao (2010) recorded that 20% of the isolates tested showed resistance to pyraclostrobin and boscalid.

CONCLUSION

The usage of single site fungicides such as boscalid extensively as a part of *B. cinerea* control strategies have to be performed accurately to reduce the risk of resistance in strawberries. According to the results obtained in the current study, *B. cinerea* resistance to boscalid was recorded widely at all locations tested, and it is recommended to reduce the number of applications of this fungicide in the same season and use it alternating with other fungicides in order to control this disease effectively. The mixture of pyraclostrobin and boscalid should not be the first option in control strategies in Egypt in order to manage boscalid resistant populations.

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حساسية عزلات البوترايتس سناريا التى تم تجميعها من الفراولة لمبيد البوسكاليد

الزهراء السيد أحمد[1]، ياسر محمد نورالدين شبانة[2] و محمد صبحى حماده[1]*
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الملخص

تعتبر مصر من الدول الرئيسية المنتجة للفراولة. العفن الرمادي الذي يسببه فطر بوترايتس سينيريا هو مرض مدمر يؤدي الي خسائر كبيرة في إنتاج الفراولة في جميع أنحاء العالم. قد تصاب الثمار في الحقل والتخزين والنقل. تعتمد أساليب إدارة العوامل الممرضة في الفراولة بشكل أساسي علي استخدام مبيدات الفطريات الوقائية. البوسكاليد هو مبيد فطري جديد واسع الطيف يستخدم للسيطرة علي فطر البوتريتس سينيريا في الفراولة. في الآونة الأخيرة تم ملاحظة نقص كفاءة العديد من المبيدات الفطرية المستخدمة في مكافحة فطر بوترايتس سينيريا فى العديد من البلدان. تهدف الدراسة الحالية إلي الكشف عن عزلات بوتريتس سينريا المقاومة للبوسكاليد في محافظات إنتاج الفراولة الرئيسية في مصر (البحيرة، الإسماعيلية، القليوبية) بين عامي 2019، 2021. تم جمع 269 عزلة واختبارها لتمييز العزلات المقاومة والحساسة عن طريق تحديد التركيز الفعال الذي يثبط 50% من نمو الفطريات. أظهرت النتائج أن 225 عزلة (83.6%) كانت مقاومة للبوسكاليد بينما 47 عزلة (16.4%) كانت حساسة. كانت هناك زيادة ملحوظة في ترددات المقاومة للبوسكاليد في عزلات بوترايتس سينريا. تراوحت قيم التركيز المثبطل 50% من نمو العزلات ل 17 عزلة حساسة من 0.0067 إلي 1.14 ميكروجرام/ مل بمتوسط 0.29 ميكروجرام/ مل. بينما تراوحت قيم التركيز المثبطل 50% من نمو العزلات ل 17 عزلة مقاومة من 2.1 الي 16.2 ميكروجرام/ مل بمتوسط 7.3 ميكروجرام/ مل. بلغ عدد العزلات المقاومة لكلا من مبيد البوسكاليد و مبيد البيروكلستروبين 192 مما يشير الى صعوبة الاعتماد على مخلوط كلا المبيدين فى برامج المكافحة